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Ionomycin-induced apoptosis of thymocytes is independent of Nur77 NBRE or NurRE binding, but is accompanied by Nur77 mitochondrial targeting

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Abstract

The induction of thymocyte apoptosis through the Nur77-mediated intrinsic pathway can be of physiological importance in the clonal deletion of autoreactive thymocytes during negative selection in the thymus and/or in thymocytes undergoing oncogenic transformation. Ionomycin treatment induces endogenous Nur77 expression as well as apoptosis and cytochrome *c* release in thymocytes. Here it is shown for the first time that in normal thymocytes undergoing apoptosis, ionomycin induces translocation of endogenous Nur77 not only to the nucleus, but also to mitochondria. Immunosuppressant FK506 inhibits Nur77 NBRE and NurRE binding activity but has no effect on thymocytes apoptosis, the subcellular localization of Nur77, or cytochrome *c* release. This indicates that thymocytes can undergo apoptosis through the intrinsic Nur77-mediated mitochondrial pathway and that the transactivation activity of Nur77 monomers or dimers is not necessary for thymocyte apoptosis.

Keywords: Thymocytes; Apoptosis; Nur77; Mitochondrial translocation; DNA-binding

1. Introduction

Nur77 (TR3, NGFI-B), a member of the steroid nuclear orphan receptor family, has been shown to play a role in apoptosis in the thymus [reviewed in [1,2], apoptosis of neuronal cells [3], and the regulation of hormone expression in the adrenal cortex [4]. As Nur77 may act as a transcription factor, three Nur77 binding elements have been identified: NBRE binding monomers of Nur77 protein [5], NurRE binding homodimers of Nur77 [6], and DR-5 binding heterodimers with proteins of the RXR family [7,8]. Nur77 can also be present on mitochondrial membrane and was reported to interact with Bcl-2 protein [9].

Constitutive Nur77 expression in mice results in massive thymocyte death and leads to decreased numbers of thymocytes and mature lymphocytes, while the expression of a dominant negative mutant of Nur77 (as well as antisense nur77) inhibits TCR (T-cell receptor)-induced apoptosis of developing T cells, suggesting a role of Nur77 in the negative selection of thymocytes [10,11]. There are two distinct mechanisms of thymocyte apoptosis, i.e. through extrinsic and/or intrinsic pathways. The intrinsic pathway involves the Bcl-2 and IAP family members, whereas the extrinsic pathway is initiated by a death receptor (Fas, TRAIL) [12,13]. Recently it was shown that adenosine can induce apoptosis of thymocytes and that this process is dependent on Bim, Bcl-2, and Nur77 [14].

In T cells, the expression of Nur77 is induced by an elevated intracellular calcium level as a result of TCR activation (mimicked by ionomycin treatment) [10,11]. It is believed that Nur77 may contribute to the extrinsic pathway by acting as a transcription factor and promoting the expression of pro-apoptotic genes such as FasL, TRAIL, and NDG1 [15–17], but the manner in which Nur77 regulates FasL or TRAIL expression is unclear, as the element responsive to Nur77 has still not been identified in the promoters of these genes. On the other hand it has been reported that in several cancer cell lines [9,18–20] and peripheral blood lymphocytes [9] the induction of apoptosis through the intrinsic pathway may depend on mitochondrial targeting of Nur77 or GR-receptors in response to an apoptotic signal [21,22]. Interaction of Nur77 with Bcl-2

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on the mitochondrial membrane leads to conformational changes within Bcl-2 and conversion of this anti-apoptotic protein to a proapoptotic one. This event enables the release of cytochrome *c* and activation of the caspases cascade [9]. This mode of Nur77 action is independent of its transactivation function. Using a transgenic mouse model it was shown that in apoptotic Nur77 transgenic thymocytes, cytoplasmic cytochrome *c* could not be detected, suggesting a negligible role of the intrinsic apoptosis pathway in thymic development [15].

Recently we showed that the induction of Nur77 by ionomycin and its NBRE binding is not sufficient to initiate the apoptotic process in a thymic lymphoma cell line [23]. Moreover, prevention of ionomycin-induced endogenous Nur77 DNA binding with the immunosuppressant FK506 results in extensive apoptosis of thymic lymphomas, suggesting that Nur77 acts rather through intrinsic pathways. In this paper we investigate the release of cytochrome *c* in apoptotic thymocytes, endogenous Nur77 expression in normal thymocytes, its cellular localization, and its ability to induce apoptosis. Additionally we studied the binding of endogenous Nur77 to its response elements (NBRE or NurRE), showing that ionomycin-induced apoptosis of thymocytes is independent of NBRE or NurRE Nur77 binding.

2. Materials and methods

2.1. Chemicals and reagents

Iscove's modified Dulbecco's medium was from Gibco; FCS was from Boehringer Mannheim; FK506 was a generous gift from Dr. M. Wasik, University of Pennsylvania (USA) or was purchased from Calbiochem; ethanol, methanol and glycerol were from POCh; NBRE oligonucleotide was from Bionovo, NurRE oligonucleotide from Integrated DNA Technologies; CREB oligonucleotide and T4 Polynucleotide Kinase were from Promega; poly(dI–dC)·poly(dI–dC) was from Amersham Biosciences; γ-32P-ATP was from New England Nuclear; ECL was from Pierce; Nonidet P-40, β-mercaptoethanol, antibiotics, ionomycin, propidium iodide, Protease Inhibitor Coctail, Triton X-100, paraformaldehyde, horse serum, Tris, PMSF and remaining chemicals were obtained from Sigma Aldrich. Antibodies: anti-Nur77, anti-CREB, anti-Hsp60, anti-histone H1 and anti-actin antibodies were from Santa Cruz Biotechnology; anti-goat and anti-rabbit horseradish peroxidase-labeled antibodies were from DAKO; anti-rabbit IgG conjugated with Alexa Fluor 488 was from Molecular Probes; anti-goat IgG and anti-mouse IgG conjugated with Cy3 were from Jackson Immuno Research; anti-cytochrome *c* antibody was from BD Biosciences.

2.2. Treatment of cells

Thymocytes obtained from C57BL/6 mice (2–3 months old) were cultured at 37 °C in 5% CO_2 in 24-well tissue culture plates, 10^6/ml, in Iscove's Modified Dulbecco's medium supplemented with 20 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. The cells were treated with 1 μg/ml of ionomycin or 40 nM of FK506.

2.3. Detection of apoptosis

The detection of apoptosis was based on DNA content evaluation with the use of propidium iodide and flow cytometry [24]. Briefly, after 22 h of treatment, the cells were washed twice with phosphate-buffered saline (PBS) containing 2.5% FCS, fixed with 70% ethanol for 30 min at 4 °C, and then, after washing with PBS, the cells were stained with propidium iodide (50 μg/ml in PBS without Ca^{2+}, $Mg2^{+}$) overnight at 4 °C. The cell suspensions were analyzed with a FACS Calibur flow cytometer (Becton Dickinson). DNA content was evaluated on the basis of FL-2 histograms using WinMDI 2.8 software. Apoptosis was quantified as the percentage of cells with hypodiploidal DNA content.

2.4. Nuclear extracts preparation

Nuclear extracts were prepared as described previously [25] with minor modifications. Cells were washed in cold PBS and suspended in three volumes of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate, and 0.2% Nonidet P-40, protease inhibitors, incubated on ice for 15 min, and centrifuged at 10,000 rpm at 4 °C for 10 min. The pellets were washed once with buffer A and resuspended in three volumes of buffer B (20 mM HEPES, pH 7.9, 10 nM KCl, 1 mM EDTA, 1 mM EGTA, 420 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors) and nuclear proteins were extracted by rocking for 30 min at 4 °C. After centrifugation (10,000 rpm, 10 min) the supernatants were stored at −70 °C in small aliquots. Protein concentrations were determined using BCA.

2.5. Electromobility shift and supershift assays

Double-stranded consensus oligonucleotides, NBRE: 5′-TCGAGTTT-TAAAAGGTCATGCTCAATTTG-3′ [18], NurRE: 5′-GATCCTAGTGATATT-TACCTCCAAATGCCAGGA-3′ [6] and CRE: 5′-AGAGATTGCCTGAC-GTCAGAGAGCTAG-3′ [26] were 5′ end-labeled with γ-^{32}P-ATP by T4 Polynucleotide Kinase. Nuclear extracts (5 μg) were incubated with binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 4% glycerol) and 0.25 mg/ml (for NBRE and CRE) or 0.03 mg/ml (for NurRE) of poly(dI–dC)·poly(dI–dC) for 15 min at room temperature. After addition of labeled oligonucleotides, the incubation proceeded for 30 min. DNA–protein complexes were resolved on 6% native polyacrylamide gel in TBE buffer (45 mM Tris–borate, pH 8.3, 1 mM EDTA). The gels were incubated overnight at −20 °C with a storage phosphor screen (Molecular Dynamics) and scanned with a Typhoon 8600 multi-imager (Molecular Dynamics/Amersham Pharmacia Biotech). The specificity of the complex formation was confirmed by performing the binding reactions in the presence of a 50-fold excess of unlabelled oligonucleotides or by 1 h preincubation of nuclear extracts with 2 ìg of anti-Nur77 (clone M210) antibody recognizing DBD domain [27–29].

2.6. Confocal microscopy

Immunofluorescence analysis was performed as described previously [30], with minor modifications. After treatment, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 37 °C and washed three times with PBS. The cells were then permeabilized with 0.15% Triton X-100 in PBS containing 10% horse serum for 1 h at room temperature. The washed cells were incubated overnight with polyclonal antibodies: anti-Nur77 or anti-CREB (at a 1:200 dilution) and anti-Hsp60 (1:20), followed by one-h staining with secondary antibodies: anti-rabbit IgG conjugated with Alexa Fluor 488 (1:200) and anti-goat IgG conjugated with Cy3 (1:400). For cytochrome *c* staining, cells were incubated with monoclonal anti-cytochrome *c* IgG followed by anti-mouse IgG conjugated with Cy3 (1:400). Antibodies were diluted in permeabilization buffer. After triple washing with PBS, the cell suspensions were placed onto microscope slides and directly subjected to visualization under a confocal microscope (Bio-Rad MRC-1024ES) with the use of a 10% laser. For each experiment, controls were performed, including the examination of samples without primary antibodies staining.

2.7. Subcellular fractionation

To obtain mitochondrial, nuclear, and cytosolic fractions we used the Mitochondria Isolation Kit. Briefly, cells were washed twice with cold PBS and lysed. After centrifugation at 800×*g* for 10 min, the nuclear pellet was washed with lysis buffer, centrifuged at 1000×*g* for 10 min, suspended in SDS sample buffer, and sonicated. The postnuclear supernatant was centrifuged at 10,000×*g*

for 15 min. The pellet containing mitochondria was dissolved in sample buffer and the clear supernatant was used as a cytosolic fraction.

2.8. Western blotting

Cell fractions were boiled and subjected to SDS-PAGE on 12% gel. The resolved proteins were transferred to PVDF membrane (Milipore). After transfer, the membrane was blocked with 1% casein in TBS and then incubated with 1 μg/ml rabbit anti-Nur77 antibody (M-210) followed by secondary horseradish peroxidase-labeled antibodies (DAKO). The bound antibodies were visualized using the ECL blotting detection system.

2.9. Measurement of cytochrome c level

Concentration of cytochrome *c* in the cytosol of thymocytes was measured by an ELISA kit (RD Systems) according to the manufacturer's procedure.

3. Results

3.1. Ionomycin treatment leads to the induction of Nur77 expression, cytochrome c release, and apoptosis of thymocytes

TCR activation with subsequent intracellular calcium level increase leads to programmed cell death of autoreactive thymocytes. Calcium ionophores, such as ionomycin, can mimic this process in vitro. In our experiments, 75–90% of cells underwent apoptosis, quantified as the percentage of cells with hypodiploidal DNA content, after 22 h treatment with 1 μg/ml of ionomycin. During this time, spontaneous apoptosis occurred in 25–40% of untreated thymocytes incubated at 37 °C (Fig. 1A) and in 0–2% of cells incubated at 4 °C (data not shown). After 2 h incubation with ionomycin we observed about 7% cells undergoing apoptosis (Fig. 1A), the same percentage of apoptotic thymocytes is observed in untreated probes (not shown). 6 h treatment with ionomycin resulted in 30% apoptosis versus about 15% without treatment. An increased level of intracellular calcium induces the expression of endogenous Nur77, a nuclear receptor thought to be indispensable for TCR-dependent apoptosis. In accordance with other authors' results, we observed high expressions of Nur77 in whole cell extracts after 2 and 6 h of incubation (Fig. 1B). This demonstrates that the consequences of in vitro ionomycin treatment in our model are consistent with a pivotal event in calcium-mediated apoptosis in thymocytes. In untreated cells, incubated at 37 °C, endogenous Nur77 protein level was insignificant and time-independent (Fig. 1B, lanes 1 and 3).

Using confocal microscopy we investigated whether cytochrome *c* is released from the mitochondria to the cytosol in thymocytes undergoing ionomycin-induced apoptosis. In untreated cells, cytochrome *c* displayed mainly a punctate, peripheral pattern of localization, corresponding to the distribution of mitochondria in thymocytes. After 6 h of ionomycin treatment, cytochrome *c* could be seen as a diffused mist around the nuclei (Fig. 1C). Release of cytochrome *c* to the cytosol was confirmed by ELISA. We managed to detect an approximately fourfold increase in the concentration of cytochrome *c* in the cytosol of the thymocytes treated for 6 h with ionomycin (Fig. 4B).

3.2. Ionomycin-induced Nur77 NBRE and NurRE binding activity is inhibited by FK506

TCR-induced Nur77 is capable of NBRE and NurRE binding. Likewise, we detected NBRE as well as NurRE binding in the nuclear extracts of ionomycin-treated thymocytes (Fig. 2A, B, lanes 2 and 4). The specificity of the complex formation was confirmed by preincubation with cold oligonucleotides (Fig. 2 lane 6, 8) and assay using antibodies raised against an epitope within DNA-binding domain of Nur77 protein (Fig. 2 lane 9). Binding of the transcription factor CREB was used as a control of protein content in the nuclear extracts (Fig. 2C).

Recently we found that FK506 was able to inhibit endogenous Nur77 DNA-binding activity in thymic lymphomas [21]. Now we studied the effect of FK506 [31,32], on endogenous Nur77 binding activity in normal thymocytes. We found that in thymocytes, as in thymic lymphomas, the immunosuppressant FK506 prevented the DNA binding of monomers of endogenous Nur77 to NBRE (Fig. 2A, lanes 3 and 5) and homodimers of Nur77 to NurRE (Fig. 2B, lanes 3 and 5) 2 h as well as 6 h after ionomycin treatment. The presence of FK506 has no influence on the DNA-binding activity of CREB (Fig. 2C).

3.3. Inhibiting NBRE and NurRE binding activity by FK506 has no effect on the mitochondrial localization of Nur77

Nur77 mitochondrial targeting as a prerequisite for the apoptotic process has been observed in numerous cancer cell lines [9,18–20] and peripheral T lymphocytes [9], but not in thymocytes so far. To ascertain this possibility, we performed confocal immunofluorescence studies on the subcellular localization of endogenous Nur77 in thymocytes treated with ionomycin alone and ionomycin with FK506. We observed co-localization (yellow) of Nur77 protein (green) and Hsp60 (red), used as a marker of mitochondria, upon 6 h of ionomycin treatment in about 30–40% of cells. Addition of FK506 did not abrogate Nur77 mitochondrial localization in contrast to DNA binding. No mitochondrial localization of CREB could be observed (Fig. 3A) under the same conditions.

For a further additional check of the intracellular localization of Nur77, we fractionated thymocytes into mitochondrial, nuclear, and cytosolic fractions. The purity of each fraction was analyzed by Western blotting with appropriate organelle markers (Fig. 3C). Using Western blotting we analyzed the expression of Nur77 in the fractionated thymocytes untreated and treated with ionomycin for 0.5, 1, 2 and 6 h. Nur77 protein is rapidly induced after 1-h incubation and present mainly in cytosol. Prolonged time of incubation results in redistribution of Nur77 to the mitochondria and nucleus. While after 6 h of treatment, Nur77 was mainly localized on the thymocyte mitochondria. FK506 did not significantly alter the localization of Nur77 (Fig. 3B).

There is no significant difference between the amount of Nur77 protein in the nucleus of thymocytes treated with FK506 and ionomycin compared with treatment with ionomycin alone. However, the differences in DNA binding by Nur77 in the presence of FK506 are clear (Fig. 2, lanes 3 and 5).

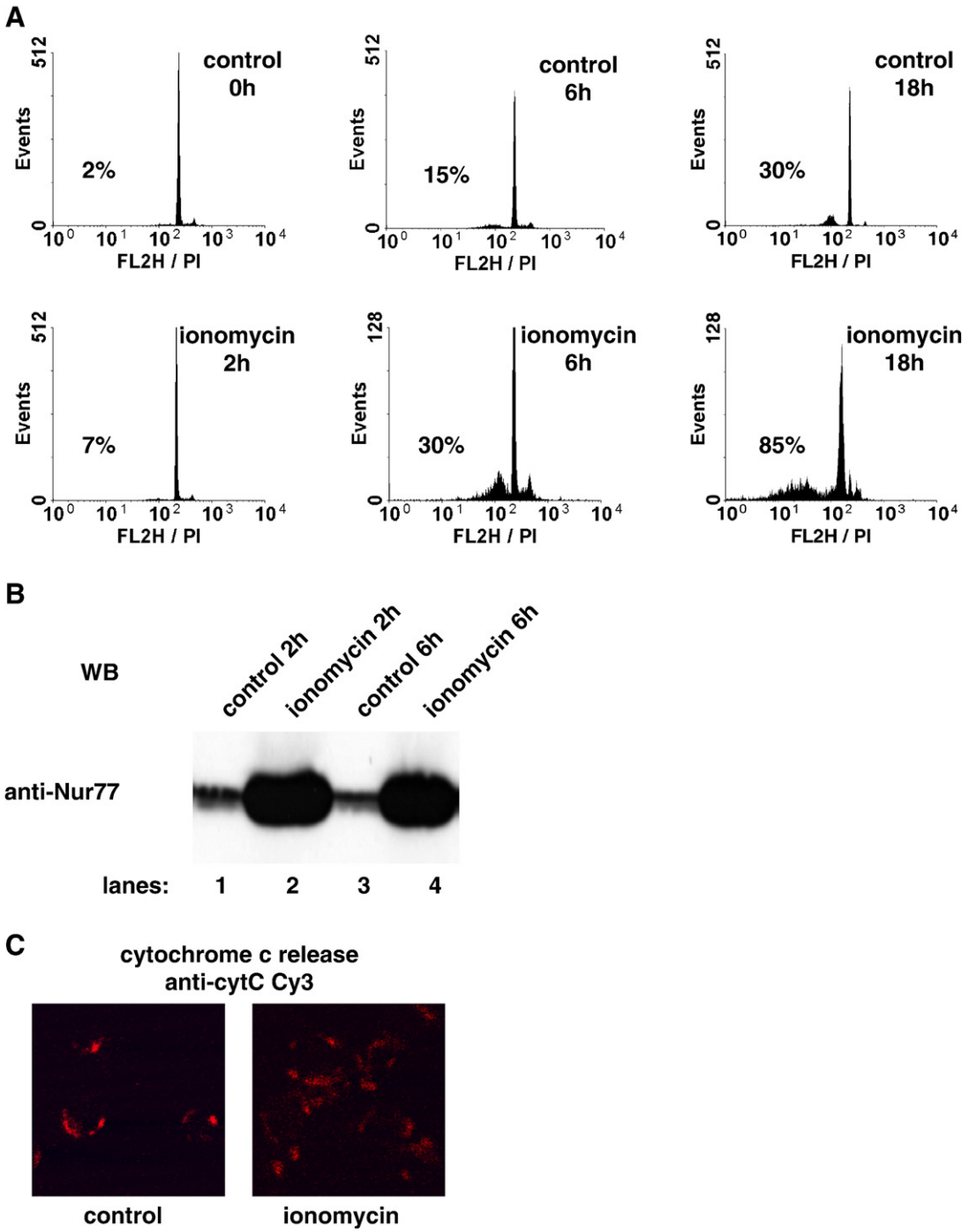

Fig. 1. Ionomycin induces thymocytes apoptosis, Nur77 expression, and release of cytochrome *c*. (A) Flow cytometric analysis of thymocytes apoptosis. Cells were treated as described for indicated time and stained with propidium iodide to visualize DNA fragmentation. Apoptosis was quantified as the percentage of cells with hypodiploidal DNA content with the use of the WinMDI program. Representative histograms are shown. (B) Western blot of Nur77 protein present in whole cell lysates from thymocytes cultured for 2 and 6 h in the presence of ionomycin or in medium only. Results representative of two experiments are shown (C) Cytochrome c release into the cytosol in response to ionomycin. Thymocytes were treated for 6 h, stained with anti-cytochrome *c* antibody and Cy3-labeled secondary antibody (red), and then examined by confocal microscopy. Representative pictures are shown.

3.4. Inhibiting of Nur77 DNA-binding activity has no effect on apoptosis and cytochrome c release

The exact mechanism of Nur77 action in the negative selection of thymocytes is controversial (DNA-binding vs. mitochondrial translocation or both). The release of cytochrome *c* to the cytosol initiates the activation of caspase-9, and subsequently caspase-3. Inhibiting DNA-binding activity by FK506 had no influence on ionomycin-induced apoptosis (Fig. 4A) and cytochrome *c* release (Fig. 4B). We detected by ELISA an approximately fourfold increase in the concentration of cytochrome *c* in the cytosol fractions of thymocytes treated for

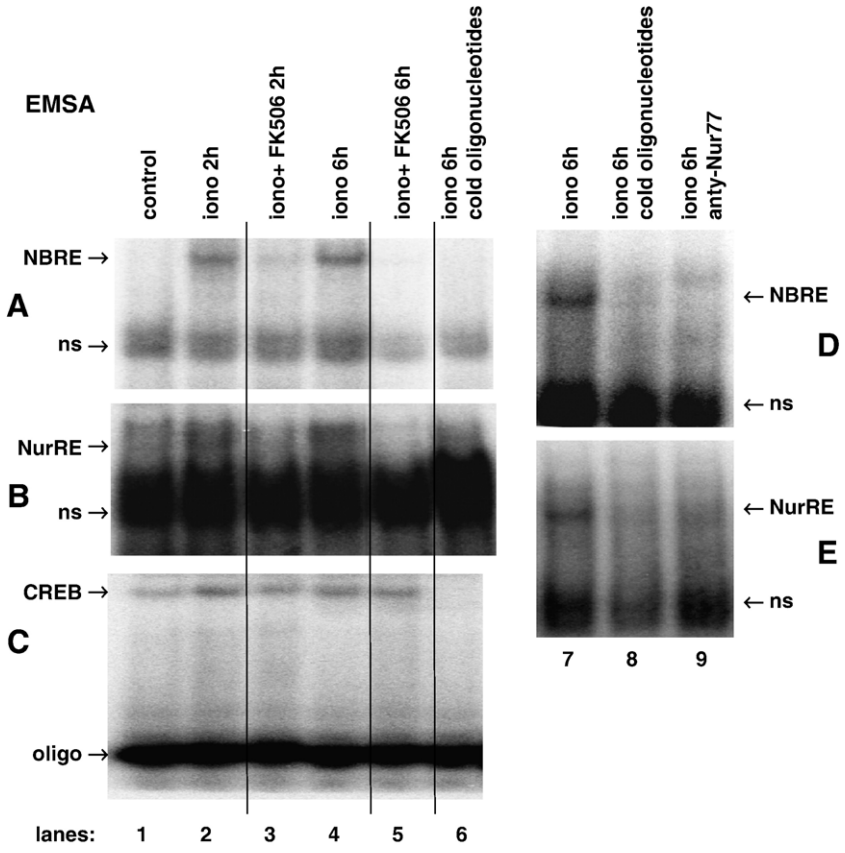

Fig. 2. Nur77 DNA-binding activity in apoptotic thymocytes. Nuclear extracts were incubated with P^{32}-labeled double-stranded oligonucleotide containing NBRE (A) or NurRE (B) and separated electrophoretically in native conditions. U—untreated cells, I—ionomycin, IFK—ionomycin+ FK506. As a control, a probe containing the CRE sequence was used (C). For NBRE and NurRE, the unbound probe was run off the gel for improved DNA–protein complex visibility and is not shown. The specificity of the complex formation was confirmed by preincubation of the nuclear extracts with a 50-fold excess of unlabelled consensus oligonucleotides (lanes 6, 8) or with anti-Nur77 antibody raised against an epitope located within DNA-binding domain (lane 9). Representative results are shown. Lines indicate image manipulation in order to crop irrelevant data.

6 h with ionomycin or ionomycin with FK506 and undergoing apoptosis. The immunosuppressant alone influenced neither the viability of the cells nor cytochrome *c* content in the cytosol.

To explain how apoptosis is induced in our model, we examined the effect of FK506. We demonstrated that FK506 inhibits Nur77 DNA binding, but not mitochondrial translocation or ionomycin-induced cytochrome *c* release (Fig. 4A) and apoptosis of thymocytes (Fig. 4B). We show here for the first time that in thymocytes, FK506 blocks DNA binding by Nur77, but prevents neither Nur77 mitochondrial targeting, nor cytochrome *c* release, nor cell death. In other words, the ability of both monomers and homodimers of Nur77 to bind DNA is dispensable for its proapoptotic function in immature T cells. Hence, mitochondrial translocation is probable mechanism of Nur77 action in the thymus.

4. Discussion

Nur77 was shown to take part in the negative selection of thymocytes. Genetic manipulation with the expression of Nur77 (constitutive expression as well as the introduction of a dominant negative mutant) resulted in an abnormal image of the thymus. Data collected to date suggest that Nur77 regulates apoptosis of T cells via its transactivation activity. Using transgenic mice with constitutive expression of Nur77, Rajpal et al. [15] reported that Nur77-mediated apoptosis in thymocytes does not involve Nur77 mitochondrial targeting and cytochrome *c* release. However, it was shown in these mice [33] that constitutive expression of Nur77 leads to a dramatic decrease (20-fold) in their thymocyte cellularities, leaving alive mostly DN (double negative) thymocytes, but lacking DP (double positive) thymocytes. It should be noted that in the normal thymus, DP thymocytes comprise about 85% of the total thymocytes and that this subpopulation of thymocytes is under negative selective pressure [34].

Rajpal et al. suggested that in thymocytes, Nur77 induces apoptosis by a Bcl2/mitochondrial-independent mechanism [15]. Several authors also observed strong correlation between Nur77 DNA-binding activity and apoptosis as well as TRAIL induction in cancer cells [16,17]. Nur77 is believed to exert its effect in cells by activating the transcription of proapoptotic genes and/or by silencing the transcription of anti-apoptotic ones [35,36], but there is no direct evidence that Nur77 acts this way. Rajpal et al. discovered novel proapoptotic genes downstream from Nur77 involved in a novel apoptotic pathway activating caspase-8 and that Nur77 could play a role as a

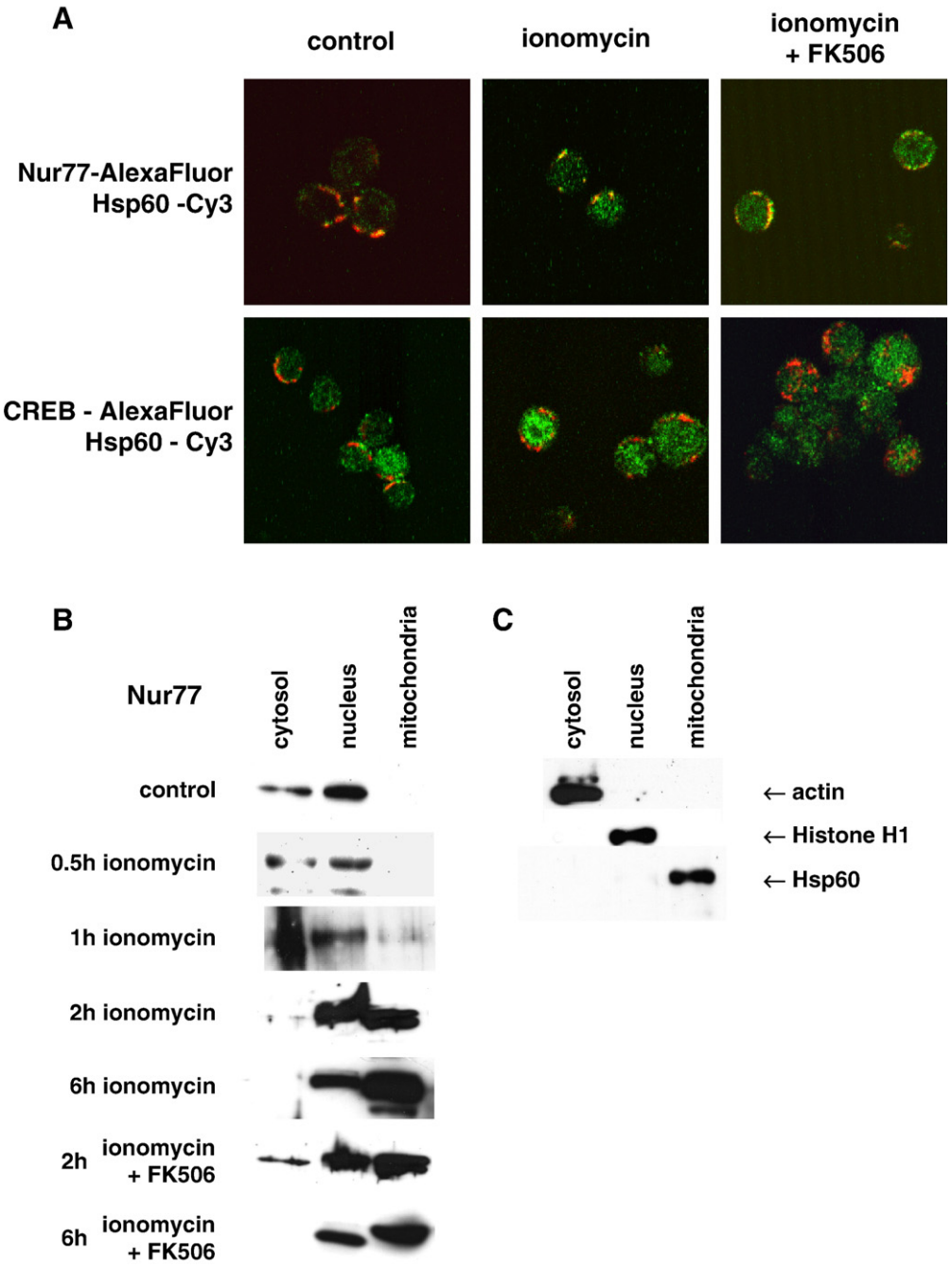

Fig. 3. Endogenous Nur77 translocation to mitochondria in response to ionomycin. (A) Cells were stimulated with either ionomycin alone or ionomycin with FK506 for 6 h. Fixed and permeabilized cells were stained for endogenous Nur77 or CREB with the use of specific antibodies followed by Alexa Fluor-labeled secondary antibody (green), and for mitochondria-specific protein Hsp60 with Cy3-labeled secondary antibody (red). The cells were then examined by confocal microscopy. Nur77 colocalized with Hsp60 (yellow), contrary to CREB used as a control. Representative results are shown as overlays. (B) Western blot analysis of the distribution of Nur77 in the cytosolic, nuclear, and mitochondrial fractions after treatment of thymocytes with ionomycin or ionomycin with FK506 for indicated time. One result of each duplicate experiment is shown. (C) Confirmation of the purity of three fractions by Western blotting with the use of antibodies specific for actin, histone H1, and Hsp60, which are specific markers of the cytosolic, nuclear, and mitochondrial fractions, respectively.

transcription factor for the known proapoptotic genes FasL and TRAIL [15]. However, it remains unclear whether these potential downstream genes are direct transcriptional targets of Nur77, as in the FasL promoter there is no consensus sequences for Nur77 binding, and in mice carrying the *gld/gld* FasL mutation or CD30 mutation [37,38], thymocyte apoptosis was not altered.

Here we have shown that in a normal thymocyte population, as in thymic lymphomas developed from HY-transgenic mice (Rapak et al., submitted), endogenous Nur77 translocates to mitochondria as well as to the nucleus upon a calcium signal. We noticed that in normal thymocytes and thymic lymphomas, Nur77 presence in the nucleus is relatively short compared with its presence on mitochondria, where Nur77 has a tendency to

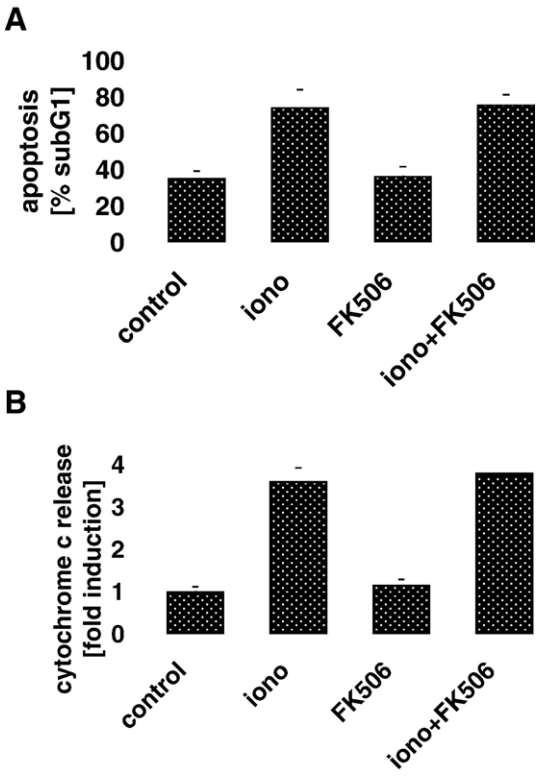

Fig. 4. FK506 influences neither thymocyte apoptosis nor cytochrome *c* release. (A) Apoptosis of thymocytes was measured as the percentage of cells with hypodiploidal DNA content after 22 h of treatment. (B) Concentration of cytochrome *c* in the cytosol of thymocytes after 6 h of incubation was measured by an ELISA kit (RD Systems) according to the manufacturer's procedure. The bars represent the average ± mean of four experiments.

accumulate over time. Additionally, Nur77 mitochondrial targeting was accompanied by cytochrome *c* release in cells undergoing apoptosis. These results strongly suggest that in T cells, as it was shown in prostate cancer and other cancer cells [9,18–20,39], Nur77 may induce apoptosis via an intrinsic pathway involving cytochrome *c* release, induction of FasL expression is rather secondary event, which takes part in enhancing apoptotic response.

In previous studies on lymphomas we found that the immunosuppressant and calcineurin [31] and JNK/p38 [32] inhibitor FK506 has the ability to inhibit Nur77 binding to the NBRE sequence [23]. We therefore applied this tool to examine the importance of Nur77 binding to NBRE or NurRE consensus sequences. We found that cytochrome *c* is released and normal thymocytes underwent apoptosis upon calcium signaling even in the absence of Nur77 binding to NBRE and NurRE sequences. This shows that Nur77 binding activity to NBRE and NurRE is not necessary for thymocyte apoptosis. On the other hand, FK506 treatment did not inhibit Nur77 mitochondrial translocation and cytochrome *c* release. This suggests that endogenous Nur77 may take a part in the regulation of thymocyte apoptosis also by the intrinsic pathway and that its transactivation activity as a monomer or homodimer is dispensable for apoptosis. Since Nur77 binding to DNA is inhibited by FK506 the transcriptional activity of Nur77 does not obviously take place. However, it is not excluded that the transactivation activity of the heterodimer of Nur77 with RXR [7,8] can also be engaged in the control of the apoptosis of thymocytes.

Altogether, our results suggest that Nur77 can contribute to the negative selection of thymocytes via translocation to the mitochondria, acting as an adaptor protein facilitating the release of cytochrome *c* to the cytosol. It is worth mentioning that it has been shown that mutant Nur77 constructs lacking a DNA-binding domain exerted stronger proapoptotic activity than the wild type of the receptor [9,18,39] by facilitated mitochondria targeting. The induction of thymocyte apoptosis through the Nur77-mediated intrinsic pathway can be of physiological importance in the clonal deletion of autoreactive thymocytes during negative selection in the thymus and/or in thymocytes undergoing oncogenic transformation. In agreement with these possibilities, we found that thymic lymphomas from mice expressing transgenic TCR autoreactive against male (HY) antigen were resistant to TCR-mediated apoptosis [40], despite of nuclear expression of Nur77 in these ionomycin-resistant lymphomas [41]. This suggests that in thymocytes apoptosis can occur through the Nur77-mediated mitochondrial intrinsic pathway and that during oncogenic transformation of thymocytes this pathway could be impaired, liberating tumor cells from host surveillance mechanisms as well as from anticancer therapies.

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